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Regulatory Mutants of Simian Virus 40: Constructed Mutants with Base Substitutions at the Origin of DNA Replication

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Mutants of simian virus 40 (SV40) with base substitutions at or near the origin of replication of the viral genome have been constructed by bisulfite mutagenesis at the *BglI* restriction site of SV40 DNA, followed by transfection of cells with the *BglI*-resistant (*BglI*^{r}) DNA so generated. Based on plaque morphology at different temperatures, the resulting *BglI*^{r} mutants could be classified into four groups. Class I mutants (designated *ar* for "altered restriction") were indistinguishable from wild-type SV40; class II mutants (designated *shp* for "sharp plaque") produced small, sharp-edged plaques; class III mutants (designated *sp* for "small plaque") produced small plaques at 32°C, 37°C and 40°C; and class IV mutants (designated *cs* for "cold sensitive") produced small plaques at 32°C and wild-type plaques at 37°C and 40°C. That the altered plaque morphology of *sp* and *cs* mutants was related to mutation at the *BglI* restriction site was demonstrated by co-reversion to wild-type of the plaque phenotype and *BglI* sensitivity. The nucleotide sequence around the original *BglI* site was determined in the DNA from one mutant of each class. In each case a different base-pair substitution was found, at a site outside sequences coding for SV40 proteins. When rates of replication of mutant DNAs were measured during productive infection, *ar* mutant DNA was synthesized at a rate comparable to that of wild-type SV40 DNA, *shp* mutant DNA was made at a rate exceeding that of wild-type, *sp* mutant DNA was synthesized at a lower rate than that of wild type, and *cs* mutant DNA synthesis was reduced at 32°C, but about the same as the wild-type rate at 40°C. These patterns of mutant DNA synthesis were unaltered in cells co-infected with mutant and wild-type virus, i.e. the defects in DNA synthesis were not *trans*-complementable. We conclude that the defective mutants have single base-pair changes in a *cis* element that determines the rate of viral DNA replication, presumably within the origin signal itself.

1. Introduction

The developmental cycle of simian virus 40 in permissive monkey cells involves sequential expression of viral genetic elements: transcription of early genes, processing and translation of early RNA, replication of viral DNA, transcription of late genes, and processing and translation of late RNA (for recent reviews, see Fareed & Davoli, 1977; Reddy *et al.*, 1978; Fiers *et al.*, 1978). At each of these steps, signals in the DNA or RNA appear to regulate the rate at which a given molecular event occurs. One way to determine the nature and function of such regulatory signals is to isolate and characterize viral mutants with altered signals. Since a physical and functional map

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of the SV40† genome and its total nucleotide sequence are now available, it is possible to identify specific regions of the genome likely to contain regulatory elements and to construct mutants with alterations within the relevant DNA segments. For this purpose mutants with single base-pair changes would be especially valuable. With this objective in mind, we have recently developed a local mutagenesis method that efficiently generates viral mutants with base substitutions at pre-selected sites in the viral DNA (Shortle & Nathans, 1978*a*). Our first application of this method, reported in this paper, has been the construction and characterization of mutants with base alterations at the origin of replication of SV40 DNA.

Replication of the SV40 genome begins at a unique site in the viral DNA, between the coding sequence for early and late genes at co-ordinate 0·67 in the standard SV40 map, and proceeds bidirectionally around the circular molecule, terminating opposite the origin (Danna & Nathans, 1972; Fareed *et al.*, 1972). The origin of replication (*ori*) appears to be a specific nucleotide sequence signal, whereas the terminus of replication coincides with the junction of the two growing forks rather than a particular sequence (Brockman *et al.*, 1975; Lai & Nathans, 1975). Analysis of deletion mutants and evolutionary variants of SV40 indicates that the *ori* sequence is within an approximately 75 base-pair segment between map co-ordinates 0·66 and 0·67 (Gutai & Nathans, 1978; Subramanian & Shenk, 1978; DiMaio & Nathans, unpublished observations). This viral DNA segment also binds tightly to SV40 T antigen (Tjian, 1978), the protein product of the early gene required for initiation of viral DNA replication (Tegtmeyer, 1972). Examination of the nucleotide sequence between 0·66 and 0·675 of a map unit reveals an array of overlapping symmetrical sequences (Subramanian *et al.*, 1977), including a 27 base-pair G + C-rich palindrome (Fig. 1), the twofold symmetry of which could be related to bidirectional initiation of replication. We therefore set out to generate mutants with base substitutions within this

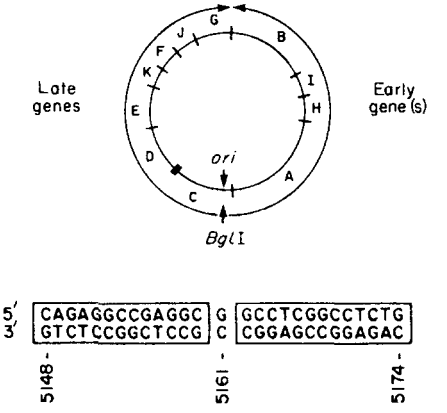


FIG. 1. A simplified map of the SV40 genome, showing the 27 base-pair symmetrical nucleotide sequence at the map position of the origin of replication (*ori*) of viral DNA. Circular arrows indicate the direction of early and late transcription and the junctions between early and late genomic segments at 0·17 and 0·67 of a map unit. Positions of *Hin*dII and III fragments A to K are noted (Danna *et al.*, 1973). The numbers below the sequence (hyphens omitted for clarity) indicate the nucleotide positions assigned by Reddy *et al.* (1978). *Bgl*I cleaves within this palindrome (Zain & Roberts, personal communication).

† Abbreviations used: SV40, simian virus 40; wt, wild-type.

palindrome. Among the resulting mutants were three phenotypically distinct groups with non-complementable defects in viral DNA replication. By correlating nucleotide changes in mutant DNAs with their rates of replication, we have identified specific nucleotide pairs that appear to be part of the *ori* signal.

2. Materials and Methods

(a) *Enzymes and gel electrophoresis*

All enzymes were purchased from commercial sources except *Bgl*I and *Alu*I, which were prepared by published procedures (Duncan *et al.*, 1978; Roberts *et al.*, 1976). Electrophoresis in agarose and acrylamide gels was carried out as previously described (Brockman & Nathans, 1974).

(b) Bgl*I-resistant SV40 mutants*

Details of the enzymatic and chemical reactions used in the construction of the *Bgl*I^r SV40 mutants described in this paper have been published elsewhere (Shortle & Nathans, 1978*a*). Stocks of mutant virus were prepared by infecting BSC-40 cells in 75 cm^2 flasks with twice plaque-purified mutant isolates.

(c) *Rates of mutant virus production*

Confluent monolayers of BSC-40 cells (Brockman & Nathans, 1974) in 16-mm microwells (about 10^5 cells) were infected at a multiplicity of 4 with 0·1 ml of diluted virus stock. After a 2-h adsorption period at 37°C, 0·9 ml of minimal essential medium plus 2% fetal calf serum (MEM-2) was added, and the dishes were transferred to the appropriate temperature (see text). At various times after infection, 1 set of infected cells was lysed by 3 cycles of freeze-thawing, and the number of plaque-forming units present in the total lysate was determined.

(d) *DNA sequencing*

Purified viral DNA cleaved with *Hin*dIII and dephosphorylated with bacterial alkaline phosphatase was labelled at 5′ termini using bacteriophage T4 polynucleotide kinase and [γ-^{32}P]ATP (New England Nuclear, 1000 to 3000 Ci/mmol) (Maxam & Gilbert, 1977). After digestion with *Hpa*II the labelled fragments were resolved on 5% (w/v) polyacrylamide gels. The desired fragment (from map co-ordinate 0·655 to 0·725) was eluted and then chemically cleaved by the method of Maxam & Gilbert (1977). In one experiment SV40 *Hin*dIII fragments were labelled instead at their 3′ termini before *Hpa*II cleavage. For this purpose 15 μg of *Hin*dIII-digested SV40 DNA, 75 μCi of [α-^{32}P]dATP (New England Nuclear, 2000 Ci/mmol), 1·2 units of DNA polymerase I from *Micrococcus luteus*, 70 mM-Tris·HCl (pH 8·0), 7 mM-$MgCl_2$ and 1 mM-2-mercaptoethanol were incubated at 25°C for 15 min in a total volume of 45 μl.

To resolve the nucleotide-specific chemical cleavage products, electrophoresis was carried out on a 10% (1:20) polyacrylamide gel (0·3 mm × 20 cm × 40 cm) in a buffer containing 8·3 M-urea, 110 mM-Tris-borate (pH 8·3), 2·2 mM-EDTA at 1700 V and 20 mA (Sanger & Coulson, 1978). Under these conditions the gel becomes sufficiently hot to denature the 12 to 13 base-pair hairpin encompassing the *Bgl*I site. (We are grateful to R. J. Roberts for detailed information on the electrophoresis procedure.)

(e) *Quantitation of viral DNA synthesis*

Confluent monolayers of BSC-40 cells in 16-mm microwells were infected at a multiplicity of 4 with 100 μl of a diluted virus stock. After a 2-h adsorption period at 37°C, 1·0 ml of MEM-2 containing 20 μCi of [^{3}H]thymidine (6·7 Ci/mmol, New England Nuclear) was added to each microwell. For those microwells labelled for more than 24 h, the [^{3}H]-thymidine medium was replaced at 24-h intervals. (Control experiments established that the concentration of ^{3}H label in the medium declines by no more than 20% in any 24-h

period during infection.) At the end of a labelling interval, the medium was withdrawn, and 0·1 ml of lysing solution (10 mM-EDTA, pH 7·5, 0·6% sodium dodecyl sulfate) containing 15,000 cts/min of ^{32}P-labelled SV40 form I DNA was added to each microwell. Viral DNA was then isolated by the procedure of Hirt (1967). To purify the viral DNA further, each sample was digested with *Bam*H-I to linearize all viral DNA forms and was then electrophoresed in a 1·4% agarose gel. After localization by autoradiography, the viral DNA band was recovered in a small slice of agarose, placed in a glass scintillation vial containing 1·0 ml water, autoclaved for 5 min, cooled to approx. 50°C, and then 9 ml of scintillation counting fluid added. The ^{32}P-labelled SV40 marker served as an internal standard for normalizing the recovery of ^{3}H-labelled viral DNA. DNA synthesis experiments in which cells were coinfected with a *Bgl*I^{r} mutant plus wild-type SV40 followed this same protocol, except for the following: (1) ^{3}H-labelling (20 μCi [^{3}H]thymidine in 0·3 ml MEM-2) was carried out as a 4-h pulse and was terminated by the addition of lysing solution; (2) 15,000 cts/min of both ^{32}P-labelled wild-type SV40 DNA and ^{32}P-labelled *ar*1026 DNA (*Bgl*I^{r}) were added to each 0·1 ml of lysing solution; and (3) viral DNA was cleaved with both *Bam*H-I and *Bgl*I to permit electrophoretic fractionation of mutant DNA (full-length linear band) from wild-type viral DNA (double band of 52% and 48% fragments).

(f) *Localization of terminus of viral DNA replication*

The distribution of [^{3}H]thymidine among the *Hin*dIII fragments of pulse-labelled form I viral DNA was determined by the method of Danna & Nathans (1972). For experiments conducted at 37°C, an 8-min pulse of 200 μCi [^{3}H]thymidine (6·7 Ci/mmol) in 2 ml MEM-2 was applied to each 10-cm dish of BSC-40 cells at 33 h post-infection; for experiments at 32°C, a 15-min pulse was used 55 h post-infection.

3. Results

(a) *Construction of* ori *mutants by local mutagenesis*

SV40 *ori* mutants were constructed by the recently described local mutagenesis method (Shortle & Nathans, 1978*a*), as outlined in Figure 2. In brief, viral DNA was nicked in one strand with endo R·*Bgl*I, which is known to cut SV40 DNA within the palindrome shown in Figure 1 (Zain & Roberts, personal communication). The *Bgl*I nick was then extended in the 5′ → 3′ direction into a gap of about five nucleotides with *M. luteus* DNA polymerase I. As seen in Figure 2, two populations of gapped molecules should be formed, depending on which strand was nicked by *Bgl*I. In the next step, the gapped DNA was treated with the single-strand-specific mutagen sodium bisulfite to deaminate exposed cytosine residues within the gap. As noted in Figure 2, the expected targets of bisulfite are C residues at positions 5154 and 5155 (C5154 and C5155) in one population of molecules, and C5161 and C5162 in the other†. Conditions were chosen so that 30% of cytosine residues in single-stranded DNA would be deaminated to uracil (Shortle & Nathans, 1978*a*), i.e. in most molecules only one of the two exposed C residues would be deaminated. After repair of the gap with DNA polymerase and treatment of repaired DNA with *Bgl*I, *Bgl*I-resistant (*Bgl*I^{r}) form II DNA was isolated by gel electrophoresis and used to transfect monolayers of BSC-40 African green monkey cells. Randomly picked plaques were surveyed for the presence of *Bgl*I^{r} mutants by infecting monolayers of BSC-40 cells with individual plaque suspensions, labelling DNA with ^{32}P. and testing the extracted ^{32}P-labelled

† Nucleotide pairs are numbered according to Reddy *et al.* (1978). In the numbering system of Fiers *et al.* (1978), position 5161 corresponds to position 0; 5162 to position L1; 5154 to position E7; and 5155 to position E6.

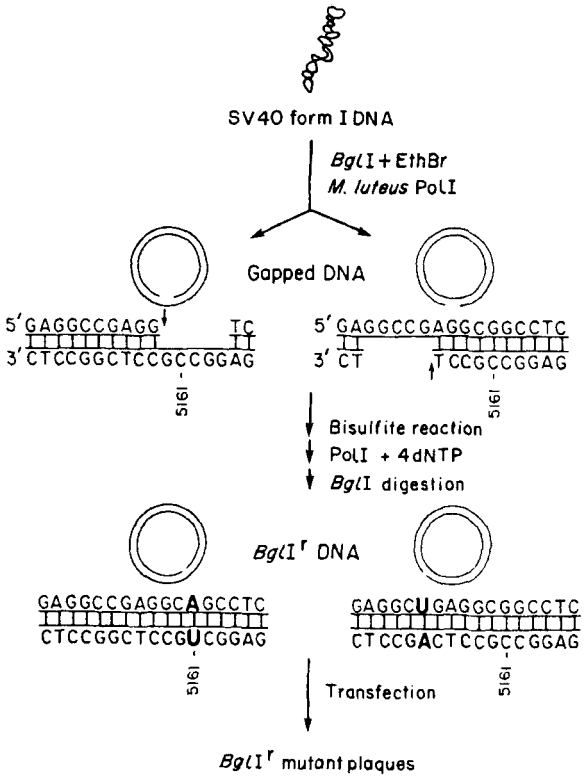


FIG. 2. Construction of SV40 mutants with base substitutions within the *Bgl*I recognition sequence of the viral DNA. See text for a description of each step in the procedure. The arrows shown in the upper sequences indicate sites of cleavage by *Bgl*I. EthBr, ethidium bromide. Hyphens have been omitted for clarity.

varil DNA for susceptibility to endo R· *Bgl*I. As illustrated in Figure 3, many plaques yielded *Bgl*I^r DNA. Of a total of 23 plaques surveyed in this way, 19 contained *Bgl*I^r mutants.

The reader may have noticed that one of the specific nicking sites for *Bgl*I given in Figure 2 is different from that found by Zain & Roberts (cited in Shortle & Nathans, 1978*b*). The sites shown in the Figure were determined by the method of McConnell *et al.* (1978). A *Hin* fragment of SV40 DNA containing the *Bgl*I site (see Fig. 1) was labelled at its 5′ ends with ^{32}P by means of polynucleotide kinase. The fragment was then cut with *Bgl*I, and the length of the resulting shorter 5′-labelled strand was precisely determined by electrophoresis in a Maxam–Gilbert (1977) sequencing gel in which the relevant sequence of the original *Hin* fragment was displayed. As seen in Figure 4, the 5′-labelled strand moves very slightly behind an oligonucleotide scored as C. Since this oligonucleotide has a 3′ phosphoryl (Maxam & Gilbert, 1977), whereas the *Bgl*I-ended strand has a 3′ hydroxyl, we presume that the slight difference in mobility is due to the presence or absence of the charged 3′ phosphoryl group (McConnell *et al.*, 1978). It should also be noted that the oligonucleotide scored as C in the Maxam–Gilbert gel has lost its 3′-terminal C residue and actually ends with

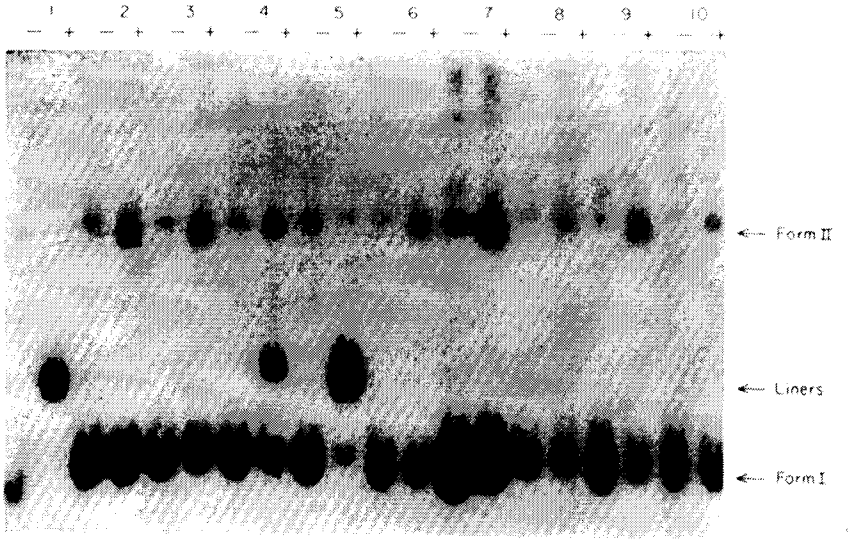


FIG. 3. Survey of plaques for *BglI*^{r} mutants. ^{32}P-labelled viral DNAs extracted from cells infected by individual plaque suspensions were subjected to electrophoresis before (odd numbers) and after (even numbers) digestion with *BglI*. Slots 1 and 2 have wild-type SV40 DNA. All but 1 of the isolates had *BglI*^{r} DNA. Unlabelled wild-type DNA present in each sample was linearized completely by *BglI*, as shown by staining the gel with ethidium bromide.

the preceeding G. Therefore *BglI* cuts this strand of SV40 DNA between residues G5159 and C5160 (Fig. 4). Similar analysis of the other strand, using 3′ end-labelled DNA, established a *BglI* scission between C5156 and T5157, as shown in Figure 4.

(b) *Plaque morphology phenotype of* BglI^r *mutants*

When plaques containing *BglI*^{r} mutants (collected initially at 37°C without regard to plaque morphology) were replated on BSC-40 cells at 32°C, 37°C or 40°C, four different phenotypes were distinguishable (Fig. 5). Class I mutants resemble wild-type virus in the size and character of plaques at all three temperatures. These were designated *ar* for "altered restriction". Class II mutants (designated *shp* for "sharp plaque") produced small plaques with sharp edges at all three temperatures, in contrast to wt plaques with indistinct borders. Class III mutants (designated *sp* for "small plaque") yielded tiny, turbid plaques at 32°C and 40°C and small plaques at 37°C. Class IV mutants (designated *cs* for "cold-sensitive") produced small plaques at 32°C, but plaques indistinguishable from wt at 37°C and 40°C. Thus on the basis of plaque morphology, mutants with base changes in the *BglI* recognition site were clearly heterogeneous in biological properties, and some were partially or conditionally defective.

(c) *Rate of mutant virus production in infected cells*

To quantitate the production of mutant virus in infected monkey cells, the rate of appearance of plaque-forming units was determined after infection of BSC-40 monolayers with a mutant or wt virus at a multiplicity of 4. As seen in Table 1, relative

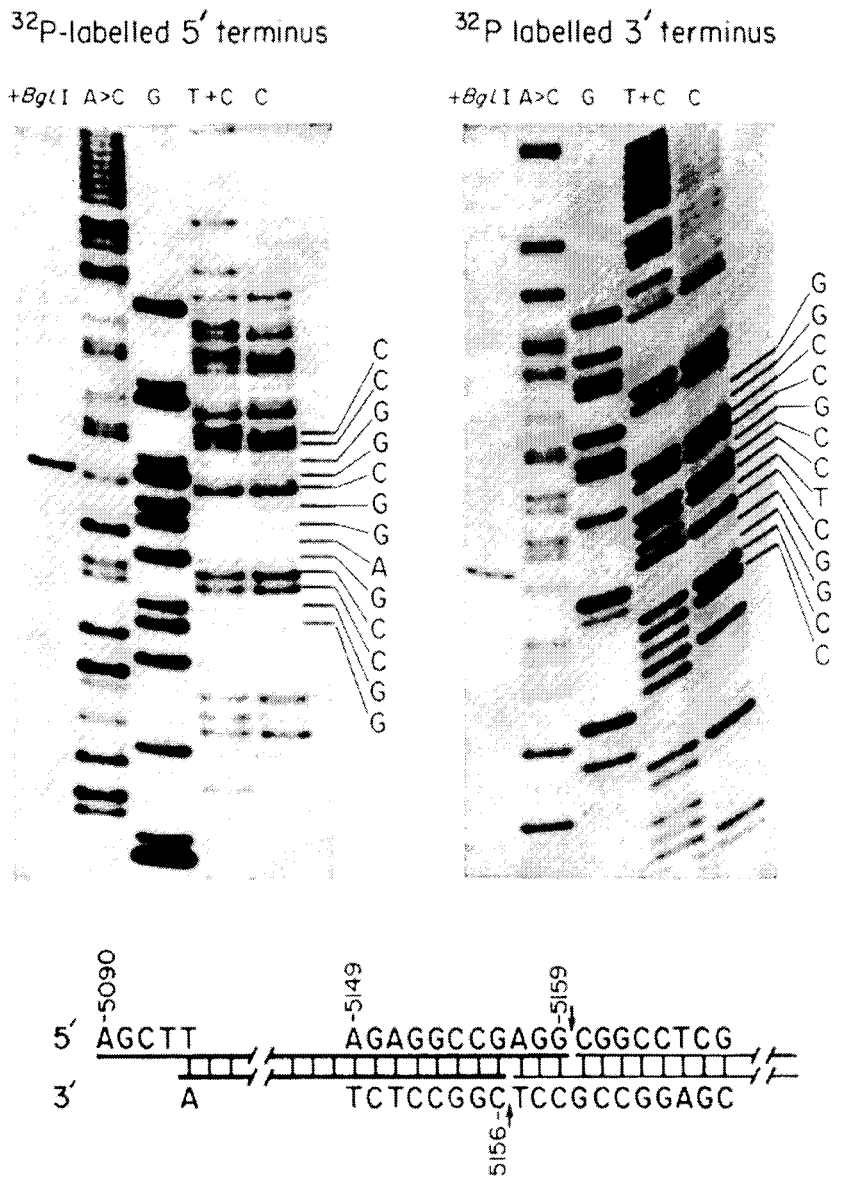


FIG. 4. Identification of the *Bgl*I cleavage site in each strand of SV40 DNA. *Left*: the *Hin*dIII/*Hpa*II fragment of SV40 DNA (between co-ordinates 0·655 and 0·73) was labelled at its 5′ ends with ^{32}P and then cleaved with *Bgl*I. The products were denatured and electrophoresed in a Maxam–Gilbert sequencing gel in which the relevant sequence of the 5′-labelled *Hin*dIII/*Bgl*I fragment is also displayed (see Materials and Methods). *Right*: the same *Hin*dIII/*Hpa*II fragment was labelled at its 3′ *Hin*dIII end with [α-^{32}P]dATP (see Materials and Methods) and then cleaved with *Bgl*I. The products were denatured and electrophoresed in a Maxam–Gilbert sequencing gel in which the relevant sequence of the 3′-labelled *Hin*dIII/*Bgl*I fragment is also displayed.

The inferred *Bgl*I cleavage sites are shown in the sequence below the electropherograms.

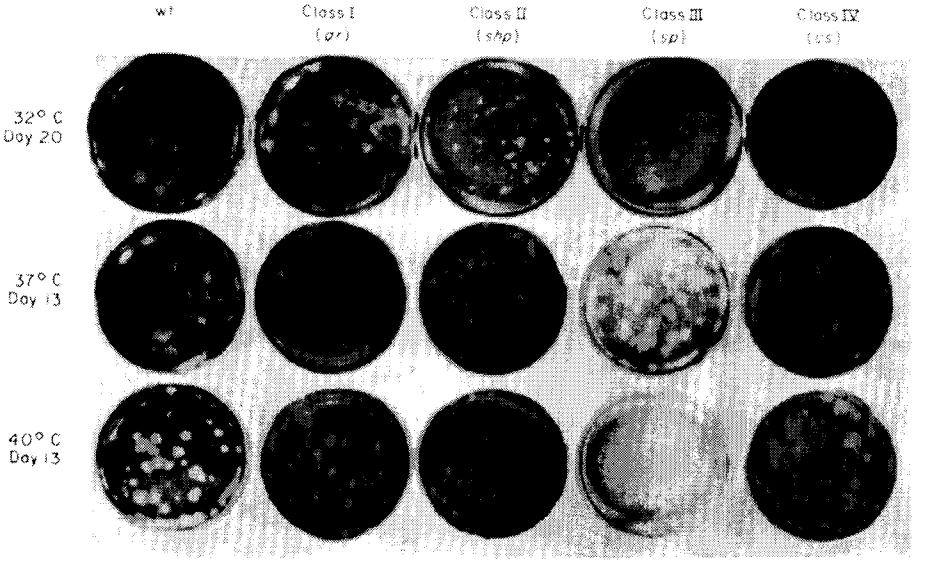


FIG. 5. Plaque morphology of *BglI*^{r} mutants.

TABLE 1

Rate of virus production by BglI^r *mutants*

Temperature	Virus	Plaque-forming units/cell			
		24 h	48 h	72 h	96 h post-infection
(°C)					
37	wt	0·13	40	380	
	*ar*1026	0·22	70	550	
	*shp*1027	0·44	60	270	
	*sp*1029	0·11	8	150	
	*sp*1030	0·20	14	250	
	*cs*1031	0·10	100	380	
32	wt		0·09	3·5	20
	*sp*1029		<0·01	0·25	1·6
	*sp*1030		0·03	0·40	2·4
	*cs*1031		0·01	0·35	3·6
40	wt	13	120	370	
	*sp*1029	0·20	28	180	
	*sp*1030	0·65	8	150	

production of virus generally correlated well with that expected on the basis of plaque size. In particular, the class I mutant (*ar*1026) was similar to wt SV40, class III mutants (*sp*1029 and 1030) showed a low rate of virus production at 32°C, 37°C and 40°C, and a class IV mutant (*cs*1031) was cold sensitive for virus production. However, with the class II mutant tested (*shp*1027) the rate of formation of infectious virus was about the same as that of wt SV40, even though class II mutant plaques are smaller than wt plaques.

In the course of these and subsequent experiments with mutant viruses, we noticed differences also in the rate of development of cellular cytopathic effects as judged by light microscopy; in particular, cells infected with an *sp* mutant showed delayed cytopathic effect at all three temperatures, and cells infected with a *shp* mutant showed accelerated development of cytopathology. Additionally, infection with an *sp* mutant caused initial proliferation of cells in the monolayer, most marked at 32°C, prior to the delayed onset of typical SV40-type cytopathic effect. Presumably these effects of *sp* and *shp* mutants are related to their respective plaque morphologies.

(d) *Map positions of mutational sites*

The change in *Bgl*I sensitivity localizes the mutational site to the *Bgl*I recognition sequence at co-ordinate 0·67 in the SV40 map. As noted earlier (Fig. 4), the *Bgl*I cleavage site in each SV40 DNA strand has been identified at the nucleotide level. However, the recognition sequence is not yet known. In order to localize the mutational sites more precisely, mutant DNA was analysed with other restriction enzymes, and finally nucleotide sequences about the original *Bgl*I site were determined for one mutant in each class.

To exclude the presence of sizable deletions at the *Bgl*I site, we initially screened each of the mutant DNAs with *Alu*I, which yields a 270 base-pair fragment (*Alu*-D) from wt DNA that includes the *Bgl*I site. In all cases *Alu*-D from mutant DNA had the same mobility as the wt *Alu*-D fragment, thus excluding the presence of deletions greater than about five base-pairs. Next we screened mutant DNA for the presence of a new *Taq*I site, since a G·C to A·T transition at position 5154 or 5168 would generate the *Taq*I recognition site $\begin{matrix}\text{T-C-G-A}\\ \text{A-G-C-T}\end{matrix}$ (see Fig. 1). Of 24 mutant DNAs surveyed, two contained new *Taq*I sites that mapped in the region of the original *Bgl*I site. Both of these mutants were of the cold-sensitive class (*cs*1031 and *cs*1032). Restriction analysis of the mutant DNAs allowed localization of the new *Taq*I site to the half of the palindromic sequence nearer the early SV40 genes. The inferred base substitution in *cs*1031 and *cs*1032 DNA, based on these results is therefore at position 5154.

To verify the inferred base-pair change in *cs*1031 and to identify the sequence alteration of the other *Bgl*I^r mutant classes, nucleotide sequence analysis of a relevant segment of DNA from one mutant of each class was carried out by the method of Maxam & Gilbert (1977). For this purpose *Hin* fragment CD (between 0·655 and 0·86 map unit) was labelled at its 5′ ends with ^{32}P and then cut with *Hpa*II (0·72 map unit). The resulting fragment with 5′–^{32}P at nucleotide A5090 (map co-ordinate 0·655) was then sequenced. The electrophoretic analysis of base-specific cleavage products is shown in Figure 6. In each case the sequence could be read up to approximately 100 nucleotides from the labelled end, i.e. well beyond the *Bgl*I site of the parental DNA. The results of these analyses, including that of wt DNA, are summarized in Figure 7. In sum, *ar*1026 shows a G·C to A·T transition at nucleotide position 5161, which is the axis of symmetry of the long palindrome; *shp*1027 has a G·G to A·T *transversion* at position 5155, within the palindrome; *sp*1030 has a C·C to A·T change at position 5162; and *cs*1031 has the anticipated C·G to T·A change at position 5154, creating the new *Taq*I site noted earlier. Thus, each of the C residues exposed to local mutagenesis (Fig. 2) was altered in one of the four mutant classes.

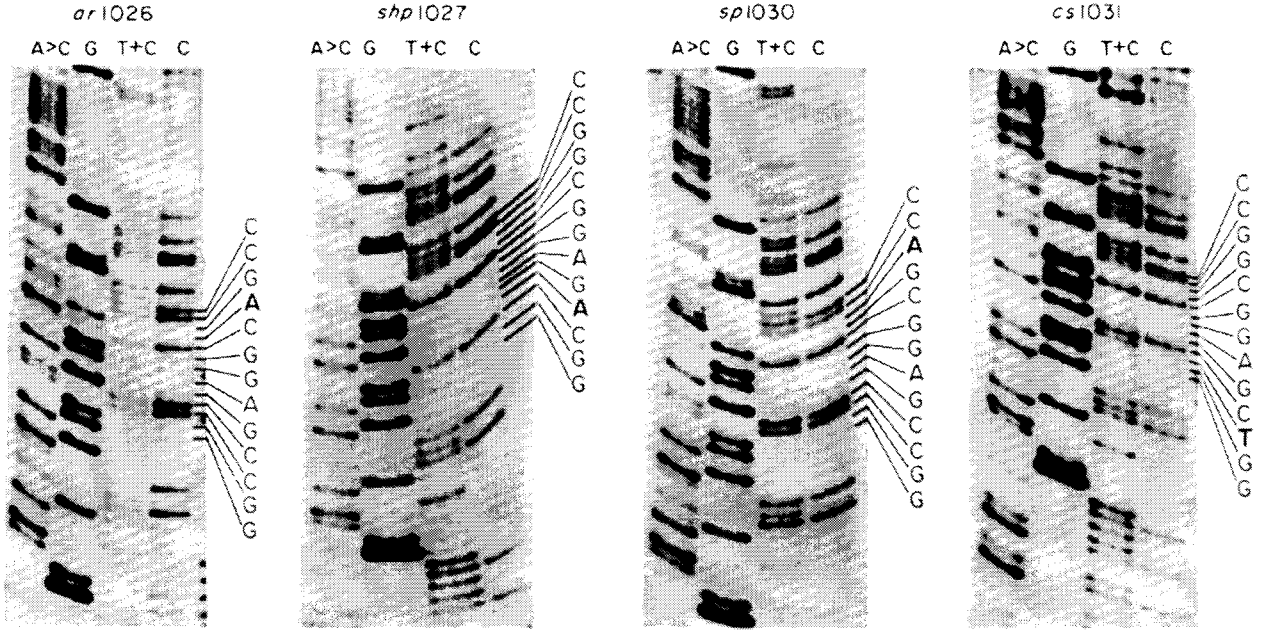


FIG. 6. Electrophoretic separation of base-specific cleavage products of the 5′ ^{32}P-labelled *Hin* CD fragments from mutant DNAs. The fragment in each case was labelled at nucleotide 5090 (0·655 map unit). The altered nucleotide is indicated in bold type.

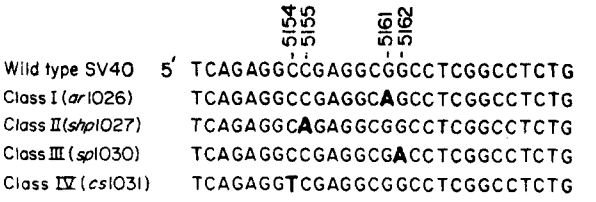


FIG. 7. Nucleotide sequence changes in *BglI*^{r} mutant DNAs. Hyphens omitted for clarity; bold type indicates the altered nucleotide.

(e) *Co-reversion of phenotype and restriction pattern*

To strengthen the conclusion that the altered phenotype of the various mutants is due to the base substitution at the *BglI* site, we looked for spontaneous revertants that produced wild-type plaques and tested their DNA for susceptibility to *BglI*. Individual mutant plaques were used to initiate virus propagation in microwell cell cultures at 32°C. In the case of *sp*1030 and *cs*1031, a large number of revertants were detectable after re-plating the microwell virus stocks. In the case of *shp*1027, however, no revertant plaques were detectable. Of six independently generated revertant plaques from the *sp*1030 series and six from the *cs*1031 series tested for sensitivity of their viral DNA to *BglI*, all were sensitive. Additionally, all six *BglI*^{s} revertants of *cs*1031 had lost the mutant *TaqI* site noted earlier. Since the plaque morphology and the restriction sites co-reverted to wild type in all instances, we conclude that the base-pair changes within the *BglI* site account for the mutant phenotypes of *sp*1030 and *cs*1031. We assume that the same holds true for the *shp* class as well.

(f) *Replication of mutant DNA*

To determine whether *Bgl*^{r} mutants had altered rates of DNA replication, viral DNA synthesis in BSC-40 cells infected with a mutant was compared to viral DNA synthesis in wt SV40-infected cells. In each case cells were infected with a single mutant, or with wt virus, at a multiplicity of four plaque-forming units per cell, and viral DNA was labelled continuously with [^{3}H]thymidine. After extraction of viral DNA at various times, the DNA was linearized with *Bam*HI (to convert all viral DNA to a single electrophoretic species), and purified by gel electrophoresis prior to assessing its radioactivity. As detailed in Materials and Methods, a fixed amount of ^{32}P-labelled viral DNA was added to the cell lysing solution in order to locate the gel position of the DNA by autoradiography, and also to normalize the ^{3}H radioactivity of each sample, thereby correcting for some variation in recovery of DNA. The results are presented in Figures 8 and 9.

As seen in Figures 8 and 9, viral DNA synthesis in cells infected by *ar*1026 was approximately equal to that in wt-infected cells; *shp*1027 DNA synthesis exceeded that of wt virus by about threefold; *sp*1029 and *sp*1030 showed a very low rate of viral DNA synthesis; and in the case of *cs*1031, viral DNA synthesis was slightly less than that of wt SV40 at 37°C. However, as seen in Figure 9, at 32°C *cs*1031 DNA synthesis was about one fifth of the rate of wt; in contrast, *sp* mutants showed no measurable temperature effect relative to wt (data not shown). Additional experiments in which viral DNA was pulse-labelled for four hours in the course of productive infection by mutant or wt SV40 gave qualitatively similar results, i.e. *ar*1026 DNA synthesis was similar to that of wt virus at 32°C, 37°C and 40°C; *shp*1027 showed

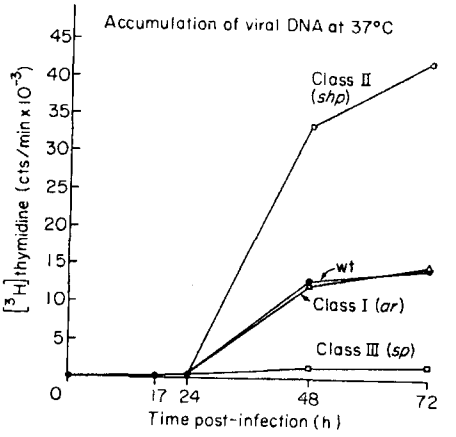


FIG. 8. Viral DNA synthesis in mutant or wt SV40 infected cells at 37°C. The following mutants were used: *ar*1026, *shp*1027, *sp*1030.

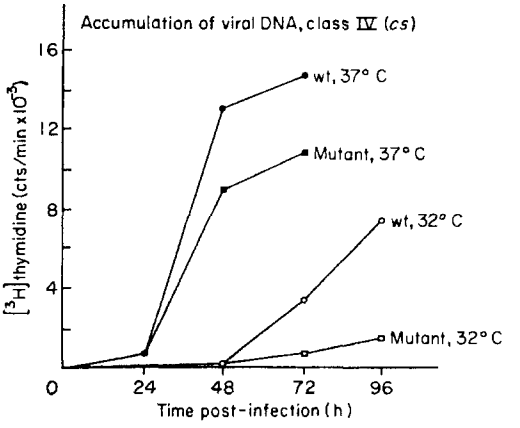


FIG. 9. Viral DNA synthesis in wt and *cs*1031-infected cells at 32°C and 37°C.

enhanced DNA synthesis relative to wt SV40 at all three temperatures; *sp*1029 and 1030 showed markedly depressed rates at the three temperatures; and *cs*1031 was cold-sensitive for DNA replication.

(g) *Direction of replication of mutant DNA*

As suggested earlier, bidirectional replication of SV40 DNA may be related to the twofold symmetry of the palindromic sequence noted in Figure 1. Since most of the *BglI*^{r} mutants no longer have perfectly symmetrical sequences within this DNA segment, we were curious to learn whether mutant DNA synthesis was still bidirectional. To determine this, we mapped the terminus of replication in pulse-labelled mutant DNA (Danna & Nathans, 1972). For this purpose mutant-infected cells were briefly exposed to [^{3}H]thymidine during active viral DNA replication at 37°C (or 32°C in the case of *cs*1031), and ^{3}H-labelled form I DNA was isolated by electrophoresis in agarose and mixed with uniformly ^{32}P-labelled wt DNA. This

mixture was then digested with *Hin*dIII and the $^{3}H:^{32}P$ ratio of each fragment determined. As seen in Table 2, composite *Hin* fragment FJGB, which contains the normal replication terminus opposite the origin (see Fig. 1), was clearly the most highly labelled with [^{3}H]thymidine in every mutant analysed, as it is in wt DNA, from which we infer that replication ends within *Hin* FJGB. (Unfortunately, pulse-labelled *sp* mutant DNA had too few counts for reliable analysis). Therefore, *ar*1026, *shp*1027, and *cs*1031 replication is still bidirectional, loss of palindromic symmetry in the latter two mutants notwithstanding.

TABLE 2

Mapping the terminus of replication of mutant DNA

Virus	Relative pulse-label in *Hin*d fragments				
	FJGB†	A	CD‡	HI	E
wt	1	0·015	0·011	0·069	0·030
*ar*1026	1	0·011	0·015	0·10	0·040
*shp*1027	1	0·016	0·014	0·07	0·034
*cs*1031 (32°C)	1	0·12	0·041	0·39	0·40
wt (32°C)	1	0·070	0·089	0·24	0·21

† Contains the site of termination of wt DNA replication (see Fig. 1). The value for this fragment was arbitrarily set at 1 and values for other fragments were normalized appropriately.

‡ Contains the origin of DNA replication.

(h) *Viral DNA synthesis in cells co-infected with mutant and wild-type virus*

Since the *Bgl*I site of SV40 DNA coincides with the map position of the origin of replication and lies outside known structural genes, it seemed likely that the DNA replication defects of at least some of the *Bgl*I^{r} mutants involve the *ori* signal itself. Such defects in a *cis*-acting element should not be complementable by wt virus functions. To test this prediction, cells were infected simultaneously with both a mutant and wt virus, each at a multiplicity of four and the incorporation of [^{3}H]-thymidine into both *Bgl*I^{r} DNA (mutant) and *Bgl*I^{s} DNA (wt) was measured during a four-hour pulse, as detailed in Materials and Methods. As seen in Table 3, each mutant

TABLE 3

Rates of mutant DNA synthesis in cells co-infected with mutant and wild-type SV40

Mutant	Percentage of wild-type rate†		
	32°C	37°C	40°C
*ar*1026	72	56	70
*shp*1027	120	146	370
*sp*1029	4·5	11	5·8
*sp*1030	16	4·4	4·1
*cs*1031	20	44	118

† ^{3}H-labelled mutant DNA/^{3}H-labelled wt DNA × 100 after incorporation of [^{3}H]thymidine between the following times post-infection: at 32°C, 48 to 52 h; at 37°C and 40°C, 24 to 28 h.

class showed its characteristic phenotype, even though wt SV40 gene products were present in the same cells: *ar*1026 DNA synthesis was similar to that of wt DNA; *shp*1027 DNA synthesis exceeded that of wt at all the three temperatures; *cs*1031 DNA synthesis was depressed relative to wt at 32°C, about one half that of wt at 37°C, and approximately the same as that of wt virus at 40°C; and *sp*1029 and 1030 DNA synthesis was depressed at all three temperatures. On the basis of these results we conclude that in each of the defective mutant classes the mutation is in a *cis*-dominant element that appears to determine the rate of viral DNA replication.

4. Discussion

The properties of SV40 *ori* mutants described in this paper are summarized in Table 4. Four different plaque morphology phenotypes form the basis of classification. A prototype mutant of each plaque morphology class was also distinguishable from mutants of the other classes by nucleotide sequence analysis and by measurements of viral DNA synthesis. However, since we have so far examined in detail only one or two mutants in each class, it is not yet clear that each phenotype is due to a unique

TABLE 4

Properties of BglI^r *point mutants*

Mutant	Base-pair change		Plaque morphology			Rate of DNA synthesis
	Position	Change	32°C	37°C	40°C	
*ar*1026	5161	G·C → A·T	wt	wt	wt	wt
*shp*1027	5155	C·G → A·T	———	small, sharp	———	>wt†
*sp*1030	5162	G·C → A·T	tiny turbid	small	tiny turbid	<wt†
*cs*1031	5154	C·G → T·A	small	wt	wt	wt at 40°C <wt at 32°C†

† Not corrected by co-infection with wt SV40.

base-pair substitution. That the mutant plaque morphology (and presumably altered DNA replication) is related to the observed base substitution at the *Bgl*I site is strongly suggested in the case of *sp*1030 and *cs*1031, by the finding that for each mutant six independent, spontaneous revertants to wt plaque morphology had all regained the *Bgl*I site. (Additionally, all revertants of *cs*1031 simultaneously had lost the mutant *Taq*I site.) Although it is not clear why *shp*1027 and 1028 did not produce detectable spontaneous revertants, we suggest it is due to the lack of selective advantage of wt revertant relative to the mutant (see Table 1).

In all four mutants analysed the base-pair substitution was in a position expected on the basis of the *Bgl*I nicking sites and the small 5′ → 3′ gap produced by *M. luteus* DNA polymerase (Shortle & Nathans, 1978*a*, and Fig. 2). The fact that we did not find any double mutants may be due to their low frequency or possibly to their inability to replicate. In three of the mutants C·G to T·A transitions were found, as expected from the specificity of bisulfite in deaminating cytosine residues. However,

*shp*1027 showed a C·G to A·T transversion. Since bisulfite is not known to cause such transversions directly (Hyatsu, 1976), possibly the transversion occurred subsequent to an initial C to U change. Examination of the sequence around the affected base-pair suggests the possibility that an intrastrand loop could form in which U at position 5155 would be opposite T5165 and thus would be correctable to A by mismatch repair.

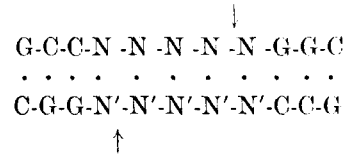
We now focus on the major experimental findings, namely that single base-pair changes at positions 5155, 5154 and 5162 affect the rate of SV40 DNA replication, whereas a change at position 5161 has little or no effect. Since these sites are outside SV40 structural genes we infer that the changes in DNA replication are not due to altered SV40-coded proteins. Supporting this inference is the observation that the defects in DNA replication were not correctable by wt SV40 gene products, indicating that the mutations are in a *cis*-acting element involved in viral DNA synthesis, presumably the *ori* signal previously mapped at this general site. Mutants of this type thus help define the nucleotide sequence comprising the replication origin. From the few mutants already characterized it is evident that a single unique nucleotide sequence at the *ori* site is not essential for viral DNA replication (since viable mutants have been isolated), and that the rate of replication of the viral replicon depends on the precise sequence at the *ori* site. Nor is exact sequence symmetry required, even for bidirectional replication. Characterization of additional, recent isolates should extend this "mutational analysis" of the SV40 *ori* site.

Our current hypothesis to explain the alterations in viral DNA replication in cells infected with *ori* mutants is based on evidence noted earlier for the involvement of the SV40 A protein (T antigen) in initiation of viral DNA synthesis (Tegtmeyer, 1972), the location of the origin of replication at or near the *Bgl*I cleavage site (Danna & Nathans, 1972; Zain & Roberts, personal communication; Shenk, 1978), and the preferential binding of A protein to the same region of the genome (Reed *et al.*, 1975; Tjian, 1978). The postulated first step in the replication of SV40 DNA is the binding of A protein to the *ori* site. We suggest that a single base-pair change in the nucleotide sequence recognized by the A protein could so alter the binding site that there is either a change in the amount of complex or in its activity. According to this hypothesis, a G·C→A·T change at position 5162 within the inverted repeat sequence leads to less efficient binding, and a C·G to A·T change at position 5155 leads to more efficient binding of A protein, whereas a change at the axis of symmetry (position 5161) has little effect. In the case of a C·G to T·A change at position 5154 (leading to cold-sensitive DNA replication) binding may be less efficient at 32°C than at 37°C or 40°C, either because of a temperature-dependent change in the structure of the binding site or as a result of a change in secondary structure of the A protein at 32°C. If a cruciform configuration of the long, 27 base-pair palindrome at the *ori* site were involved in A protein binding, a single base-pair change within the inverted repeat sequence (e.g. 5154, 5155) might lead to a marked change in stability, owing to a mismatched base-pair in each stem of the cruciform, whereas a change at or adjacent to the axis of symmetry (5161 or 5162) might not generate any mismatched pairs. However, since the 5154 G·C→A·T change leads to reduced DNA replication (at 32°C), and the 5155 C·G→A·T change leads to increased DNA replication, it seems unlikely that stability of the cruciform configuration is the primary determinant of the activity of the *ori* site. Clearly, investigation of the structure of mutant *ori* segments and of A protein binding to the *ori* site will be required to test these

possibilities. Additional insight may come from the characterization of recently isolated second-site revertants of our *Bgl*I^r mutants. These map in the *A* gene, from which we infer that they produce an A protein that recognizes the mutant DNA signal more efficiently than does the wild-type protein.

It should be noted that SV40 functions other than DNA replication may depend on signals in and around the *ori* site, and that such functions might be altered in *ori* mutants. Since the 5′ ends of both early and late messenger RNAs map in the *ori* region (Reddy *et al.*, 1978), *ori* mutations may affect rates of early and/or late transcription. Also, aside from its role in initiating DNA replication, the viral A protein inhibits transcription of the *A* gene, possibly by binding at or near the *ori* site (Tegtmeyer *et al.*, 1975; Reed *et al.*, 1976; Khoury & May, 1977). Therefore a class of mutants with base changes around *ori* might have altered autoregulation and hence overproduction of A protein. In a preliminary survey of our *Bgl*I^r mutants it was found that both *shp*1027 and *sp*1030 overproduce A protein (Lazarowitz, unpublished observations), suggesting that the *ori* binding site and the autoregulation site, while not independent, are not identical. A more detailed study of A protein synthesis and of early and late transcription by these and similar mutants is in progress.

The identification of single base-pair changes that result in *Bgl*I resistance obviously helps define the nucleotide sequence recognized by *Bgl*I. Recently two additional mutants have been isolated in which base-pair 5158 or 5159 has been altered (Shortle & Nathans, unpublished observations): in each case the *Bgl*I site is retained. Therefore the *Bgl*I recognition sequence must be interrupted by non-specific nucleotides. Combining these data with the nucleotide sequence at the *Bgl*I cleavage site of polyoma DNA reported by Friedmann *et al.* (1978), we conclude that the *Bgl*I recognition sequence and cleavage sites are as follows:



The frequency of *Bgl*I sites in a variety of DNAs is consistent with such a six base-pair recognition sequence (Roberts, 1978). It should be noted that *Bgl*I creates cohesive termini unique to a given site, as do restriction enzymes that make staggered breaks distal to their recognition sequences (Roberts, 1978).

Finally, we comment briefly on more general applications of the local mutagenesis method for the isolation of regulatory mutants of SV40. Based on the properties of *ori* mutants described in this paper, it is likely that constructed mutants with base changes in other regulatory signals (e.g., promoters, RNA processing signals, ribosome binding sites) can be detected by plaque morphology changes at different temperatures, without reliance on changes in restriction enzyme sensitivity. For example, a set of conditionally or partially defective *Bgl*I-sensitive mutants has been isolated by transfecting cells with DNA mutagenized at gaps within or adjacent to the *Bgl*I site, without first enriching for restriction enzyme-resistant molecules. Nor is selection of genomic sites for local mutagenesis limited to sequences recognized by restriction enzymes, since randomly gapped molecules of SV40 DNA can be fractionated by annealing to specific immobilized DNA fragments (Shortle *et al.*, 1979). From such gapped molecules it should be possible to construct mutants with base substitutions (or small deletions) within any pre-determined segment of the viral genome.

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Note added in proof: As indicated in the Discussion, we have recently isolated new *ori* mutants with single base-pair substitutions near the *Bgl*I site. These mutants were constructed by local mutagenesis of SV40 DNA that had been nicked at the *Bgl*I site and gapped in the $3' \rightarrow 5'$ direction by controlled digestion with *E. coli* exonculease III. The gapped, mutagenized DNA was then used directly to generate mutant plaques. Two such mutants whose DNA retained the *Bgl*I site, were cold-sensitive for plaque formation and for DNA synthesis; *cs* 1033 has a G·C to A·T transition at position 5158, and *cs* 1034 has a G·C to T·A transversion at position 5159. Thus nucleotide pairs at positions 5154, 5155, 5158, 5159 and 5162 appear to be part of the *ori* signal.

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